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Identification of a New Class of 5′-Adenylylsulfate (APS) Reductases from Sulfate-Assimilating Bacteria

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A gene was cloned from *Burkholderia cepacia* DBO1 that is homologous with *Escherichia coli cysH* encoding 3′-phosphoadenylylsulfate (PAPS) reductase. The *B. cepacia* gene is the most recent addition to a growing list of *cysH* homologs from a diverse group of sulfate-assimilating bacteria whose products show greater homology to plant 5′-adenylylsulfate (APS) reductase than they do to *E. coli* CysH. The evidence reported here shows that the *cysH* from one of the species, *Pseudomonas aeruginosa*, encodes APS reductase. It is able to complement an *E. coli cysH* mutant and a *cysC* mutant, indicating that the enzyme is able to bypass PAPS, synthesized by the *cysC* product. Insertional knockout mutation of *P. aeruginosa cysH* produced cysteine auxotrophy, indicating its role in sulfate assimilation. Purified *P. aeruginosa* CysH expressed as a His-tagged recombinant protein is able to reduce APS, but not PAPS. The enzyme has a specific activity of 5.8 μmol · min^{-1} · mg of protein^{-1} at pH 8.5 and 30°C with thioredoxin supplied as an electron donor. APS reductase activity was detected in several bacterial species from which the novel type of *cysH* has been cloned, indicating that this enzyme may be widespread. Although an APS reductase from dissimilatory sulfate-reducing bacteria is known, it shows no structural or sequence homology with the assimilatory-type APS reductase reported here. The results suggest that the dissimilatory and assimilatory APS reductases evolved convergently.

Archaea, bacteria, cyanobacteria, fungi, and plants reduce sulfate to sulfide, but they do so for different purposes. One form of the reduction pathway, termed "assimilation," is carried out by aerobic organisms and is necessary for cysteine synthesis. Another type, termed "sulfate dissimilation," is carried out by anaerobic prokaryotes, which in the absence of molecular oxygen use sulfate as a terminal electron acceptor for respiration. In assimilation, sulfide is incorporated into the thiol group of cysteine, while in dissimilation, it is released as a waste product in the form of hydrogen sulfide. Both reduction pathways, illustrated in Fig. 1, are similar in outline but different in detail. First, sulfate is activated by adenylation in a reaction catalyzed by ATP sulfurylase. In sulfate dissimilators and plants, the adenylation product, 5′-adenylylsulfate (APS), is reduced to sulfite, which is then further reduced to sulfide. In sulfate-assimilatory microorganisms, the initial substrate for reduction is not APS, but rather, it is the phosphorylated derivative 3′-phosphoadenylylsulfate (PAPS), formed after ATP-dependent phosphorylation of APS by APS kinase.

The enzymes that catalyze the first reduction step distinguish sulfate dissimilation from assimilation. In dissimilatory sulfate-reducing bacteria and archaea, an enzyme termed APS reductase (EC 1.8.99.2) (Apr) has been characterized (14, 29). It is heteromeric, composed of a flavin-containing subunit related to fumarate and succinate dehydrogenases and an iron-sulfur subunit resembling the 7Fe-type ferredoxins (14). The genes encoding the subunits have been named *aprA* and *aprB*. The source of electrons is uncertain, but may be a low potential cytochrome c_3 (11). A similar enzyme exists in sulfide-oxidizing phototrophic and chemotrophic bacteria that are able to extract electrons from sulfide for carbon dioxide fixation or ATP synthesis. In these organisms, APS reductase operates in the reverse direction, producing APS from sulfite and 5′-AMP.

Sulfate-assimilating microorganisms like *Escherichia coli* contain an enzyme termed "PAPS reductase" (EC 1.8.99.4) encoded by *cysH*, which does not share sequence homology with dissimilatory APS reductase. Because of the absolute substrate requirement of PAPS reductase, the product of the *cysC* gene (APS kinase) is necessary for *E. coli* to assimilate sulfate (17). The electron donor for PAPS reductase is thought to be thioredoxin (Trx) or glutaredoxin (Grx) (19), protein cofactors that transfer electrons from NADPH or reduced glutathione (GSH), respectively. Functional homologs of PAPS reductase have been characterized from enteric bacteria, a chemoautotrophic bacterium, fungi, and cyanobacteria (13, 26). The *cysH* family of enzymes was recently expanded with the iden-

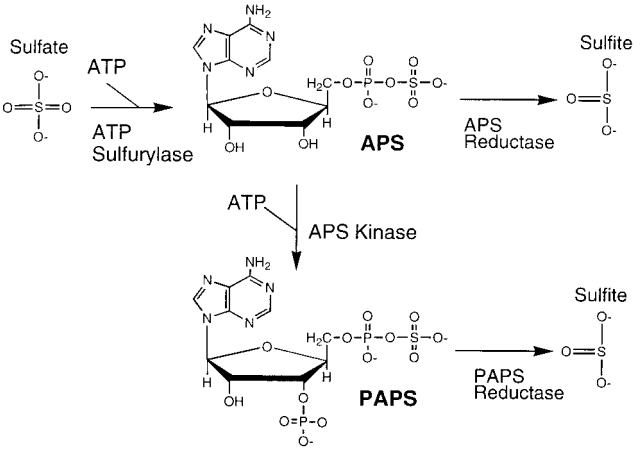

FIG. 1. Sulfate reduction pathways. The chemical name or acronym is indicated below or above the structure. The enzyme name is indicated below the arrow.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source or reference[a]
Strains		
Bacillus subtilis 168	Prototroph	BGSC
Burkholderia cepacia DBO1	Prototroph	30
Escherichia coli		
JM221 (CGSC 5745)	*cysD91*	CGSC
JM81A (CGSC 5744)	*cysC92*	CGSC
JM96 (CGSC 5746)	*cysH56*	CGSC
CBK103 (CGSC 7055)	*cysG98*::Tn*5*	CGSC
AT2427 (CGSC 4502)	*cysJ43*	CGSC
JM39 (CGSC 5043)	*cysE51*	CGSC
JM109	*lacI*^{q}	Laboratory stock
BL21(DE3)/pLysS	pET expression	Novagen, Inc.
Mycobacterium sp. strain PYO1	Prototroph	Zylstra[b]
Pseudomonas aeruginosa		
PAO1	Prototroph	15
JJDCD	*cysH*::Tc	This study
Rhizobium tropici USDA9030	Prototroph	NRGC (21)
Plasmids		
pMB1-BcysH	*B. cepacia cysH* on a 9-kbp *PstI* fragment in pMB1	This study
pUC-PaAPR	0.9-kbp *cysH* gene from *P. aeruginosa* cloned into pUC19	This study
pUC-PaAPRΔ	*P. aeruginosa cysH* insertional mutation plasmid	This study
pET-APR1	*Arabidopsis thaliana* APR1 cDNA cloned into pET30a	2
pUCP22-APR1	*A. thaliana* APR1 cDNA cloned into pUCP22	This study
pUCP22	Broad-host-range vector	31
pB-PaAPR	*P. aeruginosa cysH* cloned into pBluescript SK(+)	This study
pBluescript SK(+)	Cloning and expression	Stratagene, Inc.
pET-PaAPR	*P. aeruginosa cysH* cloned into pET30a	This study

[a] NRGC, Peter B. van Berkum, USDA-ARS National *Rhizobium* Germplasm Collection, U.S. Department of Agriculture, Agricultural Research Service, Soybean and Alfalfa Research Laboratory, Beltsville, Md.; CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.; BGSC, Daniel R. Zeigler, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio.

[b] Gerben Zylstra laboratory strain.

tification by cDNA cloning of homologs in plants (3). However, the plant enzyme differs in that it uses APS as a substrate and is able to directly use GSH as a reductant. The determinant for sulfonucleotide specificity is uncertain, but the ability to directly use GSH is probably mediated by a domain of the enzyme that functions as Grx (2, 28). PAPS reductase lacks such a domain and so requires Trx or Grx as an accessory protein.

The present study was prompted by five recent accessions to the GenBank database of bacterial *cysH* homologs that show greater amino acid sequence homology with plant APS reductase than with PAPS reductase. The *cysH* homologs from *Bacillus subtilus* (20), *Burkholderia cepacia* (this study), *Mycobacterium tuberculosis* (5), *Pseudomonas aeruginosa* (6), and *Rhizobium tropici* (18) were identified as *cysH* homologs, based on homology analysis, and none had yet been functionally characterized. Thus, the presence of APS or PAPS reductase activity was examined in a range of bacterial species, and the *cysH* homolog from *P. aeruginosa* was chosen for detailed analysis. The study revealed that a diverse group of sulfate-assimilating bacteria exist that use APS as the substrate for sulfate reduction. Until now, only the dissimilatory APS reductase was known in bacteria, but this type is structurally unlike the assimilatory-type APS reductase described here. These enzymes carry out similar reactions, but appear to have evolved separately.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and general methods. The bacterial strains and plasmids used in this study are listed in Table 1. The growth media include Luria-Bertani (LB) medium (Life Technologies, Gaithersburg, Md.) and M9 minimal medium (22) with glucose as a carbon source, mannitol for *R. tropici*, or succinate where indicated. Molecular biology techniques were performed as generally described by Sambrook et al. (23). Nucleotide sequencing was performed with the ABI PRISM DyeDeoxy Termination Cycle Sequencing System with AmpliTaq DNA polymerase (Perkin-Elmer Corp.) and an ABI 373 or 377 DNA sequencer. Nucleotide and protein sequence analyses were performed with programs from the Genetics Computer Group, Inc., package, as specified in the figure legends.

Cloning of *B. cepacia cysH*. The *B. cepacia cysH* gene was isolated in a random screen during preliminary testing of plasposons (9). Because the complete open reading frame (ORF) was not recovered during this screen, a genomic DNA fragment downstream from *B. cepacia cysH* was ligated to a kanamycin resistance marker and the pMB1 origin of replication from pTnMod-OKm (8). This plasmid DNA was transformed into *B. cepacia* and allowed to integrate into the chromosome by homologous recombination. An ~9.0-kb *Pst*I DNA fragment containing the complete *B. cepacia cysH* gene was rescued by procedures previously described (9). A partial nucleotide sequence of the rescued fragment revealed that it contains the full-length ORFs for *cysH* and *cysD*. The nucleotide sequence of both strands of the *B. cepacia cysH* gene was determined by a primer walking strategy.

Cloning and deletion of the *cysH* gene from *P. aeruginosa*. The *P. aeruginosa cysH* gene was amplified from genomic DNA by using the following PCR primers designed from the published sequence (6) and with the inclusion of *Hin*dIII and *Sac*I recognition sites 5′-GCAAGCTTACGCCGGCTTATTCCTGG-3′ and 5′-CCGAGCTCTATCGACGGTTTCAGGCC-3′, respectively. The amplified 0.9-kb DNA fragment was cloned into pUC19 and also digested with *Hin*dIII and

*Sac*I to produce the plasmid pUC-PaAPR. The clone insert was confirmed by restriction digests and partial sequence analysis.

The *P. aeruginosa cysH* locus was disrupted by digesting pUC-PaAPR with *Pin*A1, which bisects the *cysH* ORF. The 1.1-kb tetracycline resistance cassette from p34S-Tc (9) was digested with *Xma*I, and the complementary *Pin*A1-*Xma*I ends were ligated, placing the tetracycline resistance marker within the *cysH* gene. The plasmid was introduced into *P. aeruginosa* PAO by electroporation (7). After an extended outgrowth period at 42°C, the transformed cells were plated on LB medium containing 80 μg of tetracycline per ml and incubated at 30°C overnight. Since the narrow-host-range plasmid pUC-PaAPR is unable to replicate in *P. aeruginosa*, it must integrate into the chromosome in order to produce Tc^r. The recovered colonies were streaked onto M9 minimal medium, without or with cysteine. Three colonies resulting from double crossover events were isolated that proved to be cysteine auxotrophs.

The *Arabidopsis thaliana* APR1 gene was tested for the ability to complement *P. aeruginosa* PAO1 *cysH*::Tc. The APR1 cDNA was subcloned from pET-APR1 (2) as a 1.6-kbp *Xba*I-*Hin*dIII DNA fragment and ligated into the broad-host-range plasmid pUCP22 (31) which was similarly digested. The resulting plasmid, pUCP22-APR1, was constructed and maintained in *E. coli* JM109. Tight control of the *lac* promoter by *lacI*^{q} in this strain was found to be necessary due to the toxicity of the APR1 gene product in *E. coli. P. aeruginosa* PAO1 *cysH*::Tc was electroporated with pUCP22-APR1 or the parent vector pUCP22, and transformants were selected on LB medium containing 200 μg of gentamicin per ml. Both types of transformants were tested for the ability to grow at 30°C on M9-succinate medium with 80 μg of tetracycline per ml with or without cysteine. All of the pUC22-APR1 transformants were found to be prototrophic for cysteine.

Preparation of constructs for heterologous expression of CysH from *P. aeruginosa* and purification of the recombinant enzyme. The *P. aeruginosa cysH* coding sequence was amplified from pUC-PaAPR by using the following primers with *Bam*HI and *Hin*dIII sites incorporated: 5′-GGGGATCCGCCCTTTGCTACCA TTCCCGCC-3′ and 5′-GGAAGCTTCAGGCCTTGCTGATCAGGTTGC-3′. The PCR product was cloned into the same sites of pBluescript SK(+) to produce pB-PaAPR. One clone was completely sequenced on both strands. Nine nucleotide substitutions were found, resulting from either PCR-derived changes or due to sequence polymorphisms. All of the nucleotide differences were silent with respect to the reported amino acid sequence of the gene product (6). pB-PaAPR was tested for the ability to complement a range of *E. coli cys* mutants. Electrotransformed colonies were isolated on LB medium containing 100 μg of ampicillin per ml. Growing colonies were then replica plated onto M9-glucose medium with or without cysteine and incubated for 48 h at 30°C.

The gene insert from pB-PaAPR was subcloned into pET30b with *Bam*HI and *Hin*dIII. The resulting plasmid, pET-PaAPR, was transformed into *E. coli* BL21(DE3)pLysS, and colonies were selected in LB medium with 40 μg of kanamycin per ml and 34 μg of chloramphenicol per ml. A transformed 1-liter culture was grown in liquid LB medium with 40 μg of kanamycin per ml and 34 μg of chloramphenicol per ml at 30°C to an optical density at 600 nm of 0.6. The culture was then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by overnight incubation. All subsequent procedures were carried out at 4°C, and all centrifugations were at 13,800 × *g* for 10 min. The cell suspension was harvested by centrifugation and was resuspended in 100 ml of 50 mM Tris HCl (pH 8.0). The culture was sonicated on ice and centrifuged, and the supernatant was filtered through a 0.45-μm-pore-size filter. Solid ammonium sulfate was added to 20% (wt/vol) saturation, and the mixture was stirred on ice for 20 min followed by centrifugation. The supernatant was collected, solid ammonium sulfate was added to 80% saturation, and the solution was stirred for 20 min. The precipitate was collected as before, and the pellet was dissolved in 50 ml of 50 mM Tris-HCl (pH 8.0) with 1 M ammonium sulfate. Insoluble material was removed by centrifugation and then passage of the supernatant through a 0.45-μm-pore-size filter before loading onto a 50-ml phenyl Sepharose column at a flow rate of 2 ml per min. The proteins were eluted with a 250-ml linear gradient of ammonium sulfate from 1.0 to 0 M, prepared in 50 mM Tris-HCl (pH 8.0), and at the same flow rate. The peak of enzyme activity, collected between 0.2 and 0.1 M ammonium sulfate (~25 ml), was pooled, and the buffer was changed to 50 mM Tris-HCl (pH 8.0)–100 mM NaCl (buffer A) by several rounds of ultrafiltration with a YM10 Amicon membrane. The sample (~50 ml) was stirred with 2 ml (bed volume) of Ni-agarose (Talon; New England Biolabs, Inc.) for 2 h. The resin was washed twice with 20 ml of buffer A and then again with buffer A containing 10 mM imidazole. The protein was eluted in buffer A with 125 mM imidazole. The imidazole was removed by buffer exchange with an Amicon YM10 membrane. The typical yield was ~15 mg of protein from 1 liter of culture.

Enzyme assays. APS and PAPS reductase activities were measured as described previously (28). [^{35}S]PAPS (New England Nuclear, Inc.; 57.7 × 10^3 Bq · nmol^{-1}; 0.45 nmol · μl^{-1}) was used to produce [^{35}S]APS by dephosphorylation with 3′ nucleotidase (Sigma, Inc.; N8630). Unless stated otherwise, the reductase assays were done with a volume of 100 μl containing 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 5 mM dithiothreitol (DTT), 10 μM *E. coli* Trx, 25 μM [^{35}S]APS or [^{35}S]PAPS, and experimental enzyme. In some experiments, the reductant or sulfonucleotide substrates were varied as described in the figure and table legends. Sulfonucleotides were used in enzyme assays at a specific activity of 500 Bq · nmol^{-1} when present in the reaction mixture at greater than 0.66 μM or at a specific activity of 2,500 Bq · nmol^{-1} when present at between 0.10 and 0.66 μM. Initial rate conditions were maintained by varying the incubation times so that the amount of product formed was at least fivefold above the background, but no more than 10% of the substrate in the reaction. A reaction without added reductant served as the background rate. All enzyme assays and kinetic experiments were repeated at least three times. Kinetic data sets were analyzed by least-squares nonlinear regression (4). *E. coli* thioredoxin reductase was provided by C. Williams (Veterans Affairs Medical Center, Ann Arbor, Mich.). *E. coli* Grx1 and plant APS reductase APR1 were prepared as described previously (2). Other components were purchased from Sigma, Inc., including *E. coli* Trx (T3658), ferredoxin (F3013), and ferredoxin-NADP$^+$ oxidoreductase (F0628). The activities of ferredoxin and ferredoxin-NADP$^+$ oxidoreductase were measured as described previously (32).

Nucleotide sequence accession number. The GenBank accession number of the sequence reported in this publication is AF170343.

RESULTS

Identification of a *cysH* homolog from *B. cepacia* and analysis of homology. During optimization of a plasposon tagging method (9), a randomly isolated DNA clone from *B. cepacia* was identified with two tandem ORFs. The upstream ORF showed homology to *E. coli cysH*, and the downstream ORF showed homology to *E. coli cysD*, a subunit of ATP sulfurylase. The relative positions of the ORFs suggested that they could be part of an operon. A similar arrangement of genes has been reported in *R. tropici* (18). The *cysH* gene contains 250 codons, predicted to produce a 27,881-Da protein. A BLASTP search of the GenBank database revealed that the amino acid sequence encoded by *B. cepacia cysH* is most similar (67% over a 172-amino-acid stretch) to that encoded by the *cysH* homolog from *R. tropici* (18). *B. cepacia cysH* is the most recent GenBank accession of bacterial *cysH* homologs that show greater homology with plant APS reductase (28) than with PAPS reductase. A dendrogram constructed with different members of this group illustrates the sequence relationships between the enzymes (Fig. 2). PAPS reductases from fungi, cyanobacteria, enterobacteria, and a chemolithotroph, *Thiocapsa roseopersicina*, cluster in the top portion of the diagram. Clustered at the bottom are the uncharacterized bacterial *cysH* gene products and the APS reductases from plants.

An amino acid sequence alignment of PAPS reductases from *E. coli* and yeast, plant APS reductase, and the *B. cepacia* and *P. aeruginosa* CysH homologs illustrates the primary structural similarities between these enzymes (Fig. 3). All five show dispersed homology over their lengths and contain conserved motifs known as a modified PP motif (indicated with dots in Fig. 3) and a carboxyl-terminal stretch with the sequence ECGLH (indicated with asterisks in Fig. 3). The PP motif is a version of the P loop and is thought to function in nucleotide binding (24). The carboxyl-terminal motif was shown to be essential for catalytic function of the *E. coli* CysH enzyme (1). The *B. cepacia* and *P. aeruginosa* CysH enzymes and plant APS reductase show subtle differences from the PAPS reductases. Most notable are two areas that include four cysteine residues (indicated with circles in Fig. 3). The CysH homologs from the other bacteria, *R. tropici*, *B. subtilis*, and *M. tuberculosis*, are also conserved at these positions, as are all of the plant APS reductases that have been cloned to date. It should be noted that the plant enzyme contains a carboxyl-terminal extension, not shown in Fig. 3, that functions as a Grx (2). Neither the PAPS reductases nor the novel class of CysH homologs contain this domain.

Analysis of the substrate specificity of bacterial *cysH* homologs. The finding that the products of some bacterial *cysH* homologs are closely related to plant APS reductase prompted an exploration of the catalytic function of these enzymes. A survey was carried out to determine the type of sulfonucleotide reductase present in various prokaryotic species and focusing

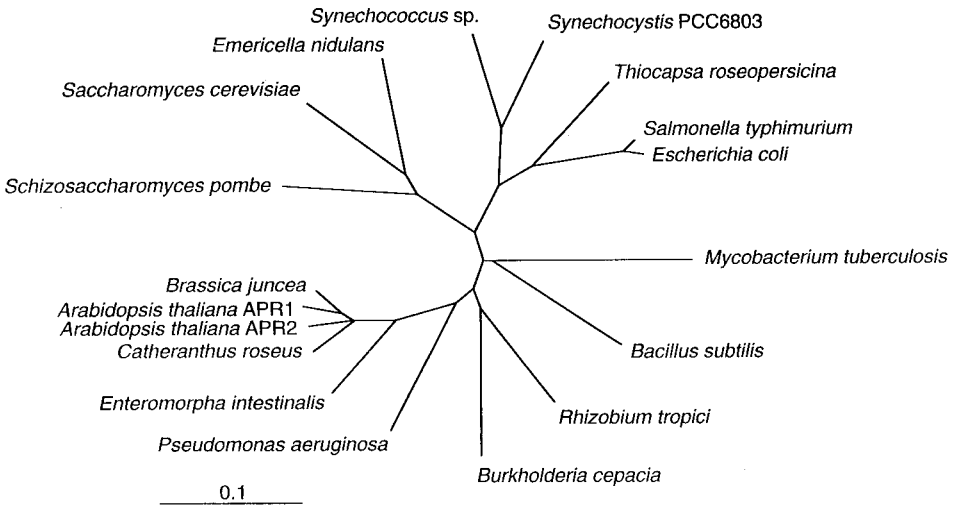

FIG. 2. Dendrogram showing the amino acid sequence relationship between different CysH homologs. The sequences were aligned by using the PileUp program, and the tree was constructed with the Paup program. The sequences were obtained from the following GenBank accession numbers: *Arabidopsis thaliana*, AF016282 (APR1) and AF016283 (APR2); *Enteromorpha intestinalis*, AF069951; *Catheranthus roseus*, U63784; *Rhizobium tropici*, AJ001223; *Burkholderia cepacia*, AF170343; *Pseudomonas aeruginosa*, U95379; *Mycobacterium tuberculosis*, Z81368; *Bacillus subtilis*, U76751; *Saccharomyces cerevisiae*, J05591; *Schizosaccharomyces pombe*, Z69729; *Thiocapsa roseopersicina*, Z23169; *Salmonella typhimurium*, M23007; *Escherichia coli*, M23008; *Emericella nidulans*, X82555; *Synechococcus* sp., M84476; and *Synechocystis* sp. strain PCC6803, P72794.

on those from which *cysH* homologs have been cloned. Cell lysates were assayed for APS or PAPS reductase activity from cultures grown on minimal medium with sulfate as the sole sulfur source. The results are shown in Table 2. As has been reported (17), *E. coli* and *Salmonella typhimurium* reduce PAPS, and the activity is stimulated by addition of Trx to the assay. In contrast, a *cysH* mutant of *E. coli* expressing plant APS reductase shows APS reductase activity that is not stimulated by Trx, also as previously reported (28). Extracts from most of the experimental bacteria reduced APS primarily, the exceptions being *B. subtilis* and *Mycobacterium* sp., which have both activities. In each case, the activity is stimulated by Trx. None of the bacterial species showed significant sulfonucleotide reductase activity when grown on cysteine-containing medium, indicating that the *cysH* gene is probably repressed by cysteine as it is in *E. coli* (17).

In vivo analysis of *P. aeruginosa* CysH. Since *P. aeruginosa* CysH is most closely related to plant APS reductase, it was chosen for detailed analysis. The question of whether *P. aeruginosa* CysH is involved in assimilatory sulfate reduction was addressed by creating a gene knockout mutant. The strain carrying the deletion was auxotrophic for cysteine, confirming the identity of *P. aeruginosa* CysH as an assimilatory-type APS reductase. The *cysH*::Tc mutant could be functionally complemented with the APR1 cDNA encoding a plant APS reductase (not shown), further illustrating the functional similarity of the plant and *P. aeruginosa* enzymes.

P. aeruginosa cysH was tested for the ability to complement *E. coli* mutations in each of the genes required for sulfate reduction, including *cysD* encoding a subunit of ATP sulfurylase, *cysC* encoding APS kinase, *cysH* encoding PAPS reductase, *cysJ* encoding a subunit of sulfite reductase, and *cysG* encoding an enzyme required for synthesis of siroheme, the prosthetic group of sulfite reductase. The *P. aeruginosa* gene was able to complement both *cysC* and *cysH*, but none of the others (not shown). The complementation result is consistent with the idea that the *P. aeruginosa cysH* product uses APS rather than PAPS, since it is able to bypass the *cysC* step in *E. coli*. The hypothesis is further supported by the sulfonucleotide reductase activity in the *E. coli cysH* mutant expressing *P. aeruginosa cysH*, which showed APS reductase activity dependent on Trx (Table 2). The growth rates on minimal medium of *E. coli cysH* and *cysC* mutants expressing *P. aeruginosa cysH* were similar, irrespective of whether cysteine was provided in the medium (not shown). This verifies the role of these enzymes in cysteine biosynthesis and confirms that cysteine auxotrophy is effectively complemented by the *P. aeruginosa cysH* product.

In vitro analysis of *P. aeruginosa* CysH. The *P. aeruginosa cysH* gene was amplified by PCR and cloned for expression as a heterologous protein. The affinity-purified preparations were extremely pure, as determined by staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis electropherograms with Coomassie blue (not shown). The pure enzyme showed Trx-dependent APS reductase activity, but no activity was detected with PAPS (Fig. 4A). The enzyme was most active with Trx as an electron donor (Table 2), showing a V_{max} of 5.8 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$. The activity was ~5-fold lower with DTT, ~70-fold lower with *E. coli* Grx1 or dithionite, and 580-fold lower with lipoic acid. No activity could be detected with ferredoxin.

The conditions for optimal activity of *P. aeruginosa* CysH were studied. PAPS reductase and plant APS reductase have different requirements. For example, plant APS reductase has a pH optimum of ~8.5, while PAPS reductase has a pH optimum of ~8.0. Also, plant APS reductase activity is markedly enhanced by sodium sulfate. Testing of *P. aeruginosa* CysH revealed that its pH optimum is ~8.5 (Fig. 4B) and its activity is not stimulated by sodium sulfate (Fig. 4C). Thus, *P. aeruginosa* CysH resembles plant APS reductase with respect to pH optimum, but not with respect to salt preference.

Initial velocity measurements of *P. aeruginosa* CysH were carried out to determine the kinetic constants and to compare its activity with that of *E. coli* CysH. Figure 5A shows the plots of $1/v$ versus 1/[APS] at various fixed concentrations of Trx. From the vertical and horizontal intercepts of the line obtained with saturating Trx, the apparent V_{max} was 5.8 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ and the $K_{m[APS]}$ was 1.75 μM. From the vertical intercepts of the lines in Fig. 5A and an independent,

FIG. 3. Amino acid sequence alignment of several CysH homologs. GenBank accession numbers of the sequences are given in Fig. 2. The sequences were aligned with the PileUp program, and the alignment was embellished with the PrettyBox program to show homologous residues. Each sequence was given an equal vote weight for derivation of a consensus sequence. Solid boxes indicate amino acid residues that are identical to the consensus at that position. Boxes with intermediate and light shading represent amino acids with similarity to the consensus at that position. ar, *A. thaliana* APR2; pa, *P. aeruginosa* CysH; bc, *B. cepacia* CysH; ec, *E. coli* CysH; sc, *S. cerevisiae* Met16. Conserved motifs are indicated with dots and asterisks. Residues conserved with plant APS reductase are indicated with circles. Only the *B. cepacia* CysH sequence is shown from the initiator methionine. The amino terminus of each of the others is not shown for the sake of brevity, nor is the carboxyl-terminal domain of APR2.

Trx titration experiment (Fig. 5B), the apparent K_m for Trx is 19.6 μM. The reaction product 5′-AMP was found to be a competitive inhibitor with respect to APS at a saturating Trx concentration, as shown by the intersection of lines on the vertical axis of the reciprocal plot (Fig. 5C). The $K_{i[5'\text{-AMP}]}$ value, calculated from the slopes of the reciprocal plot, is 1.0 mM (Fig. 5D). Although a comprehensive analysis of the kinetic mechanism of *P. aeruginosa* APS reductase has yet to be carried out, it is evident from this study that the *P. aeruginosa* APS reductase has kinetic properties similar to those described for *E. coli* PAPS reductase (1). It is therefore possible that these enzymes function via a similar catalytic mechanism.

DISCUSSION

In this study, the catalytic function of the CysH enzyme from *P. aeruginosa* was studied. The genetic and enzymological evidence is consistent with the hypothesis that this enzyme functions as a Trx-dependent APS reductase involved in sulfate assimilation.

That *P. aeruginosa* CysH catalyzes APS reduction is supported by the in vitro activity of the purified recombinant enzyme. The specificity of the enzyme for APS explains why the *P. aeruginosa cysH* gene is able to complement *E. coli cysH* and *cysC* mutant strains. The simplest explanation for complementation of *cysC* is that it is able to bypass APS kinase due to its altered substrate specificity. Plant APS reductase is also able to complement *E. coli cysC* (28). Another hypothesis is that the enzyme is a bifunctional APS kinase/PAPS reductase. However, this is unlikely, because Mg ATP, necessary for APS phosphorylation, is not supplied in the in vitro reaction mixture.

Mutation of *P. aeruginosa cysH* produced cysteine auxotrophy. Moreover, a plant APS reductase cDNA was able to

TABLE 2. P(APS) reductase activity in several bacterial species[a]

Species or strain	Activity (pmol · min^{-1} · mg^{-1}) with substrate(s):			
	PAPS	PAPS + Trx	APS	APS + Trx
Escherichia coli	37	178	1	8
Salmonella typhimurium	12	88	2	2
E. coli cysH/APR1	3	5	221	225
E. coli cysH/PaAPR	4	7	207	336
Pseudomonas aeruginosa	5	11	20	204
Pseudomonas putida	23	32	22	104
Burkholderia cepacia	2	5	15	117
Ralstonia pickettii	5	7	21	169
Rhizobium tropici	23	25	35	278
Mycobacterium sp.	17	38	33	105
Bacillus subtilis	14	67	4	10

[a] Bacterial cultures were grown on M9 minimal medium with only sulfate as the sulfur source. APR1 is an *A. thaliana* cDNA (28). PaAPR is the *P. aeruginosa cysH* gene expressed from pB-PaAPR. P(APS) reductase activity was measured with 5 mM DTT ± 200 μM Trx as the electron donor and ~0.5 μg of cell lysate protein. Incubation was at 30°C for 20 min.

complement the mutant. These results indicate that *P. aeruginosa cysH* is necessary for the synthesis of cysteine. Since the gene product catalyzes a reaction in sulfate assimilation necessary for cysteine synthesis, it can be concluded that *P. aeruginosa cysH* encodes an assimilatory APS reductase. The *P. aeruginosa cysH* gene exists as a lone ORF and does not appear to exist in an operon. A *cysB* homolog is located downstream, but it is located on the opposite strand and is transcribed convergently with *cysH* (6). Therefore, it is unlikely that polar effects resulting from the insertional mutation can account for cysteine auxotrophy. Moreover, there are no other ORFs with strong homology to *E. coli cysH* in the genome of *P. aeruginosa* PAO, which has been nearly completely sequenced, indicating that PAPS reductase or another alternate route for sulfate assimilation probably does not exist in *P. aeruginosa*.

The species survey presented in Table 2 suggests that the ability to reduce APS is widespread among aerobic, sulfate-assimilating bacteria. Until now, this activity was known with certainty only from anaerobic sulfate-dissimilating bacteria. The dissimilatory-type APS reductase shows homology with succinate and fumerate reductases (14), while the assimilatory type described here is related to the CysH superfamily. It is therefore likely that the two classes of enzyme, which carry out similar catalytic reactions, may have very different evolutionary origins. Insight into the structure and function of the CysH superfamily comes from the crystal structure of *E. coli* PAPS reductase (Protein Data Bank ID code 1SUR [24]), showing that it belongs to the adenine nucleotide α-hydrolase family, which includes ATP pyrophosphatase and the CysD-type ATP sulfurylases (24). The adenine nucleotide α-hydrolases, including assimilatory APS reductase, contain a PP motif involved in nucleotide binding. Thus, the CysH superfamily appears to consist of closely related enzymes with different substrate specificities. The finding that prompted this study was that the sequences of several newly accessioned CysH homologs are more closely related to plant APS reductase than to the known PAPS reductases. The sequence analysis in Fig. 3 showed that all of the enzymes in the CysH superfamily are remarkably similar. The only major obvious differences are two short stretches containing four cysteine residues in the plant APS reductase-type sequences that are lacking in *E. coli*, *S. cerevisiae*, and other confirmed PAPS reductases. It was initially tempting to speculate that these sequences are the determinants for APS as a substrate. However, this idea is not certain, because PAPS reductase activity was measured from *B. subtilis* (Table 2), an organism with a CysH resembling plant APS reductase. Further analysis is required to determine whether the CysH of *B. subtilis* is an APS or PAPS reductase and what role the conserved cysteine residues play in catalysis.

A comparison of *P. aeruginosa* CysH, *E. coli* PAPS reductase, and plant APS reductase revealed that other than substrate specificity, the bacterial enzymes are very similar. Both the *E. coli* and *P. aeruginosa* enzymes are not stimulated by sodium sulfate, and both lack the Grx-like carboxyl domain of the plant enzyme. Therefore, both require Trx as a source of electrons and display very similar $K_{m[\mathrm{Trx}]}$ values (1). Based on kinetic studies similar to those carried out with *P. aeruginosa* APS reductase and reported here (Table 3), a ping-pong mechanism has been proposed for *E. coli* PAPS reductase (1). It was proposed that the electrons from Trx are transferred to and stored by the enzyme for use in the second reaction step, PAPS reduction. Since PAPS reductase does not contain prosthetic

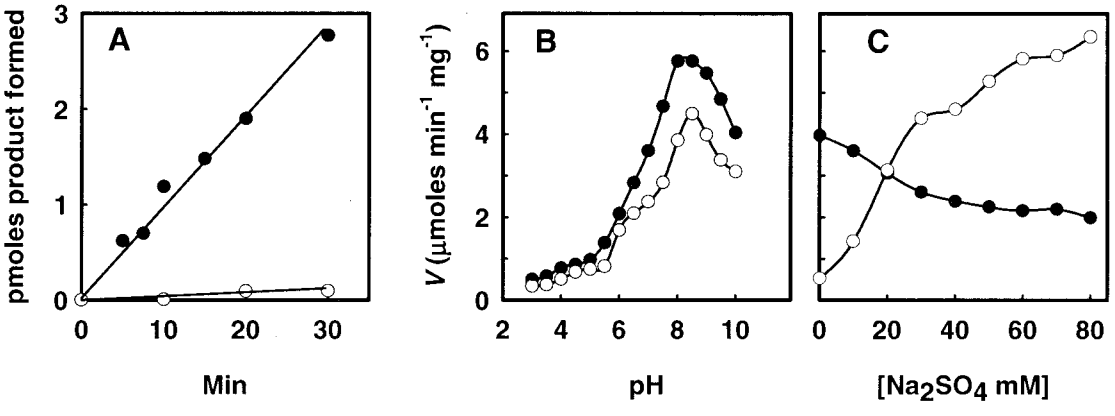

FIG. 4. Activity of pure *P. aeruginosa* CysH. (A) Activity with APS (●) or PAPS (○). The reaction mixtures contained 0.02 ng of enzyme and were incubated at 30°C for various times. (B) Activity of *P. aeruginosa* CysH (●) or plant APS reductase (○) at various pH values. The reactions were carried out at the specified pH with 50 mM MesNaOH at pH 5.5 and lower and 50 mM Tris-HCl between pH 6.0 and 10.0. The reaction mixtures with plant APS reductase also contained 500 mM sodium sulfate. All contained 0.35 ng of protein and were incubated at 30°C for 20 min. (C) Activity of *P. aeruginosa* CysH (●) or plant APS reductase (○) with various sodium sulfate concentrations. The reactions were carried out with the specified sodium sulfate concentration. The reaction mixtures contained 0.35 ng of protein and were incubated at 30°C for 20 min. The vertical axis in panel C is identical to that in panel B.

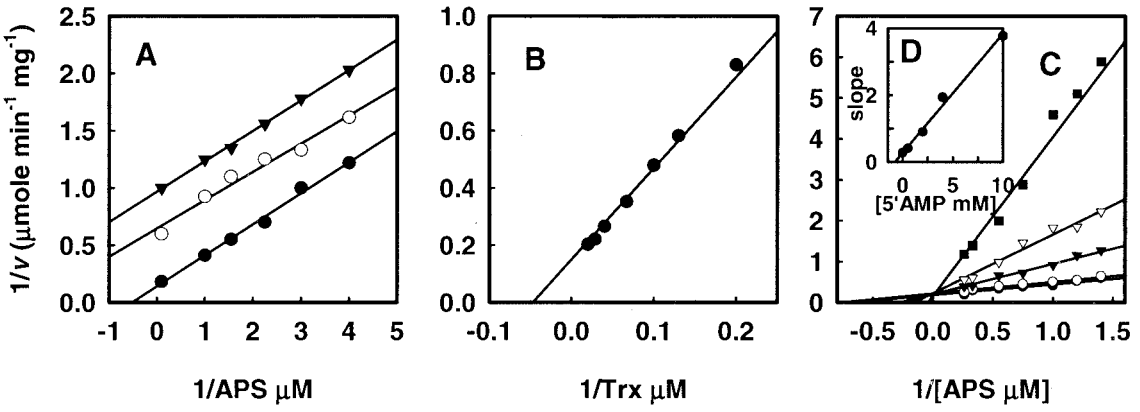

FIG. 5. Reciprocal plot analysis of *P. aeruginosa* CysH (*y* axis represents 1/*v* [micromoles per minute per milligram of protein] in plots A, B and C). (A) The concentration of APS was varied at different fixed concentrations of Trx: 50 μM (●), 10 μM (○), and 5 μM (▼). (B) The concentration of Trx was varied with APS fixed at 25 μM. (C) The concentration of APS was varied without 5′-AMP (●) or with different fixed concentrations of 5′-AMP: 0.5 mM (○), 2.0 mM (▼), 4.0 mM (▽), and 10.0 mM (■). (D) Replot of $slope_{1/APS}$ versus [5′-AMP], with data taken from panel C. The reductant in these reactions was a combination of 200 μM Trx, 1 U of thioredoxin reductase, and 0.2 mM NADPH. All reaction mixtures contained 0.35 ng of *P. aeruginosa* CysH and were incubated at 30°C. Incubation times were varied, as described in Materials and Methods.

groups or chromophores, the mechanism for electron storage is enigmatic, but was proposed to involve the reduction of a cysteine residue. The finding that *P. aeruginosa* and *E. coli* CysH enzymes share similar kinetic properties suggests that they may function via a similar catalytic mechanism. In contrast, the sulfonucleotide specificity, pH optimum, and 5′-AMP inhibition are properties that *P. aeruginosa* CysH has in common with plant APS reductase. This is a significant finding, since until now, APS reductase activity in bacteria was thought to be limited to the dissimilatory type from sulfate reducers and archaea that use it for anaerobic sulfate respiration.

Although yeast, *E. coli*, and *S. typhimurium* use PAPS for sulfate reduction, other studies have shown that APS is used for assimilatory sulfate reduction in red, brown, and green algae and vascular plants. This was thought to indicate a correlation between APS reduction and organisms containing chloroplasts. The finding that *P. aeruginosa*, a nonphotosynthetic bacterium, uses APS rather than PAPS for reduction indicates an important evolutionary divergence of bacterial CysH enzymes. A comparison of the *E. coli* and *P. aeruginosa* CysH enzyme sequences and biochemical characteristics suggests that they probably have a common structure and function via similar kinetic mechanisms. The divergent substrate requirement may be mediated by only a few amino acid residues. A future focus will be to identify these residues. A further question to be addressed is the reason for the adaptation of a PAPS-Trx system in some classes of bacteria and the prevalence of an APS-dependent reduction pathway in others.

The finding of an APS reductase in some sulfate-assimilating bacteria—*P. aeruginosa* in particular—suggests that these organisms do not require PAPS for sulfate assimilation. *P. aeruginosa*, *R. tropici*, and *M. tuberculosis* contain ORFs homologous with *nodP* and *nodQ* of *Rhizobium meliloti* (10, 16; GenBank accession no. CAA97752). These genes encode the subunits of a bifunctional ATP sulfurylase/APS kinase capable of APS and PAPS synthesis from sulfate and ATP (25). If APS is required for sulfate assimilation, what could be the function of PAPS in these organisms? The question has been most carefully studied with *R. meliloti* and *R. tropici*, where PAPS is known to be involved in the synthesis of sulfated oligosaccharides necessary for formation of a symbiotic root nodule with legumes (10, 25). Thus, some of the activated sulfate in these species is channeled into a separate pathway that leads to incorporation of sulfate into organic compounds. In this regard, it may be significant to note that *M. tuberculosis* produces sulfated glycolipids known as sulfatides (12). Thus, APS reductase may represent a key enzyme for the division of the reduction and sulfation pathways.

TABLE 3. APS reductase activity of *P. aeruginosa* CysH with various reductants[a]

Reductant	Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
10 mM DTT	1.2
10 mM DTT + 1 μg of *E. coli* Trx	5.8
10 mM GSH	0
10 mM GSH + 10 μg of Grx1	0.09
10 mM Lipoic acid	0.01
10 mM Dithionite	0.08
10 μg of ferredoxin + 1 U of FNR[b] + 0.2 mM NADPH	0

[a] APS reductase activity was measured with the indicated reductants. The reaction mixtures contained 0.35 ng of protein and were incubated at 30°C for 20 min.

[b] FNR, ferredoxin $NADP^+$ oxidoreductase.

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